Research, Society and Development, v. 9, n. 8, e603986251, 2020
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v9i8.6251

Avaliação da citotoxicidade, genotoxicidade e toxicidade subcrônica de diésteres p-metoxicinâmicos extraídos de *Copernicia prunifera* (Miller) H.E. Moore em modelo experimental

Assessment of cytotoxicity, genotoxicity and subchronic toxicity of *p*-methoxycynnamic diesters extracted from *Copernicia prunifera* (Miller) H. E. Moore in experimental model

Evaluación de la citotoxicidad, genotoxicidad y toxicidad subcrónica de diésteres p-metoxicinámicos extraídos de *Copernicia prunifera* (Miller) H. E. Moore en modelo experimental

Recebido: 02/07/2020 | Revisado: 06/07/2020 | Aceito: 06/07/2020 | Publicado: 20/07/2020

Natália do Vale Canabrava
ORCID: https://orcid.org/ 0000-0002-4841-8989
Universidade Estadual do Ceará, Brasil
E-mail: canabravanatalia@gmail.com
José Ytalo Gomes da Silva
ORCID: https://orcid.org/ 0000-0002-8697-1718
Universidade Estadual do Ceará, Brasil
E-mail: ytalogomes93@gmail.com
Bruno Bezerra da Silva
ORCID: https://orcid.org/ 0000-0002-9881-201X
Universidade Estadual do Ceará, Brasil
E-mail: brunoxbezerra@gmail.com
Marcelo Oliveira Holanda
ORCID: https://orcid.org/ 0000-0002-3125-6610
Universidade Estadual do Ceará, Brasil
E-mail: marceloh.nutri@gmail.com
Carla Laine Silva Lima
ORCID: https://orcid.org/ 0000-0002-0694-6281
Universidade Estadual do Ceará, Brasil
E-mail: carla_lainne@hotmail.com

Eridan Orlando Pereira Tramontina Florean
ORCID: https://orcid.org/ 0000-0002-5033-8670
Universidade Estadual do Ceará, Brasil
E-mail: eridan.pereira@uece.br
Icaro Gusmão Pinto Vieira
ORCID: https://orcid.org/ 0000-0002-3333-8396
Universidade Estadual do Ceará, Brasil
E-mail: icarogpv@uol.com.br
Márcia Maria Mendes Marques
ORCID: https://orcid.org/ 0000-0001-7661-2570
Universidade Federal de Picos, Brasil
E-mail: marciammm2003@gmail.com
Maria Izabel Florindo Guedes
ORCID: https://orcid.org/ 0000-0002-4569-8663
Universidade Estadual do Ceará, Brasil
E-mail: carla_lainne@hotmail.com

Resumo

Os diésteres p-metoxicinâmicos do pó de cera de carnaúba (PCO - C) - derivado do ácido cinâmico - foram associados a novas propriedades, como efeitos hipolipidêmicos, hipocolesterolêmicos e hipoglicêmicos em camundongos. O presente estudo teve como objetivo investigar o perfil de segurança da PCO - C, avaliando a toxicidade oral subcrônica, genotoxicidade e mutagenicidade usando modelos experimentais *in vivo*. Portanto, para a presente pesquisa, caracterizada como experimental e de natureza quantitativa, foram utilizados 80 camundongos *Swiss*, de ambos os gêneros, para a análise de mutagenicidade, genotoxicidade, análises dos perfis bioquímico, hematológico e toxicológico. O PCO - C não induziu alterações na massa de animais, além de não interferir nos parâmetros sanguíneos. PCO - C não mostrou hemólise de eritrócitos de camundongo. O PCO - C não causou efeitos mutagênicos ou genotóxicos em camundongos machos e fêmeas tratados com 500 mg / kg / dia durante 90 dias. Além disso, a PCO - C não alterou alguns parâmetros séricos importantes (alanina aminotransferase, aspartato aminotransferase, colesterol total e creatinina), perfil hematológico, disposição estrutural dos rins, coração, estômago e baço. Os resultados demonstram que o PCO - C não é genotóxico e não apresentou toxicidade quando usado por

via oral por até 90 dias. No geral, o estudo demonstrou a segurança do PCO - C para possíveis usos em áreas biomédicas.

Palavras-chave: Cera de carnauba; Diésteres *p*-metoxicinâmicos; Toxicidade oral subcrônica; Micronúcleos.

Abstract

P-methoxycinnamic diesters from carnauba wax powder (PCO - C) – cinnamic acid derivate – have been associated with new properties such as hypolipidemic, hypocholesterolemic and hypoglycaemic effects in mice. The present study aimed to investigate the safety profile of PCO - C by evaluating the subchronic oral toxicity, genotoxicity and mutagenicity using *in vivo* experimental models. Therefore, for the present research, characterized as experimental and of a quantitative nature, 80 Swiss mice, of both genders, were used for the analysis of mutagenicity, genotoxicity, analysis of the biochemical, hematological and toxicological profiles. PCO - C did not induce changes in the mass of animals besides not interfering in blood parameters. PCO - C showed no hemolysis of mouse erythrocytes. PCO - C caused no mutagenic or genotoxic effects in male and female mice treated with 500 mg/Kg/day during 90 days. In addition, PCO - C did not change some important serum parameters (alanine aminotransferase, aspartate aminotransferase, total cholesterol and creatinine), hematological profile, structural arrangement of the kidneys, heart, stomach and spleen. The results demonstrate that PCO - C is not genotoxic, and did not present toxicity when used orally for up to 90 days. Overall, the study has demonstrated the safety of PCO - C for potential uses in biomedical areas.

Keywords: Carnauba wax; P-methoxycinnamic diesters; Subchronic oral toxicity; Micronuclei.

Resumen

Los diésteres P-metoxicinámicos del polvo de cera de carnauba (PCO-C), derivado del ácido cinámico, se han asociado con nuevas propiedades como los efectos hipolipidémicos, hipocolesterolémicos e hipoglucémicos en ratones. El presente estudio tuvo como objetivo investigar el perfil de seguridad de PCO - C mediante la evaluación de la toxicidad oral subcrónica, genotoxicidad y mutagenicidad utilizando modelos experimentales *in vivo*. Por lo tanto, para la presente investigación, caracterizada como experimental y de naturaleza cuantitativa, se utilizaron 80 ratones suizos, de ambos sexos, para el análisis de mutagenicidad, genotoxicidad, análisis de los perfiles bioquímicos, hematológicos y

toxicológicos. PCO - C no indujo cambios en la masa de los animales además de no interferir en los parámetros sanguíneos. PCO - C no mostró hemólisis de eritrocitos de ratón. PCO - C no causó efectos mutagénicos o genotóxicos en ratones machos y hembras tratados con 500 mg / Kg / día durante 90 días. Además, PCO - C no cambió algunos parámetros importantes del suero (alanina aminotransferasa, aspartato aminotransferasa, colesterol total y creatinina), perfil hematológico, disposición estructural de los riñones, el corazón, el estómago y el bazo. Los resultados demuestran que PCO - C no es genotóxico y no presentó toxicidad cuando se usó por vía oral durante un máximo de 90 días. En general, el estudio ha demostrado la seguridad de PCO - C para usos potenciales en áreas biomédicas.

Palabras clave: Cera de carnauba; Diésteres p-metoxicinámicos; Toxicidad oral subcrónica; Micronúcleos.

1. Introduction

The Brazilian Northeast is predominantly dominated by the caatinga biome and it is recognized as one of the richest biodiversity on the planet. However, its potential is still underexplored (Albuquerque et al., 2007). Among the plants that have excellent industrial, pharmaceutical and biotechnological potential, it is possible to mention the Carnauba (*Copernicia prunifera* (Miller) H. E. Moore). This plant belongs to the family *Arecaceae* and it has as primary source of the carnauba wax (CW) (Ayres et al., 2008). In addition, CW is widely used in the food, cake, chocolate and beverage industry due to its stabilizing and conservation properties (European Food Safety Authority, 2012).

The carnauba wax has several chemical constituents, among them are the esters, free alcohols, aliphatic acids, triterpenoids and aromatic acids (Lorenzi et al., 2010; Vandenburg & Wilder, 1970). Cinnamic acids and their derivatives are characterized by phenolic compounds pharmacologically active and promising due to their high therapeutic potential as neuroprotective (Ola et al., 2015), antihypertensive (Theodotou et al., 2016), antioxidant (Freitas et al., 2016), hypocholesterolemic (Arruda-Filho et al., 2017), hypoglycemic (Rodrigues et al., 2014) effects.

In this context, Guedes et al. (2011) analyzed diesters of *p*-methoxycinnamic acid extracted from the carnauba wax powder (powder-eye - when extracted from young leaves), named as PCO - C, observed pharmacological activities of this compound in dyslipidemic mice.

Posteriorly, Rodrigues et al. (2014) evaluated the therapeutic effect of PCO - C using two different doses (100 and 150 mg/kg/body weight) in the treatment of diabetic mice induced by alloxan and observed a similar and even better reduction in glycemia than the standard drug (Glibenclamide) in diabetic mice.

Freitas et al. (2016) reported that PCO - C presented high thermal stability, ultraviolet absorption and relevant antioxidant activity (107.27 ± 3.92 mM Trolox/g of dry weight) before and after simulated *in vitro* gastrointestinal digestion and 32.46% bioaccessibility. In addition, in tests performed on human cells, PCO - C (250 mg / mL) was able to inhibit the intracellular production of reactive oxygen species.

Finally, Arruda-Filho et al. (2017) evaluated the pharmacological activity of PCO – C *in vivo* experiment of acute and chronic dyslipidemia and reported a reduction in total cholesterol and triacylglycerol after treatment with referred material.

The number of chronic non-transmissible diseases worldwide and their severe consequences for health systems in several countries, in which an estimated 40 million people die each year due to complications pathologies such as cardiovascular diseases, respiratory diseases, diabetes and cancers be reduced by implementing policies combat risk factors including tobacco smoking, high blood pressure and obesity (WHO, 2017).

Develop new drugs effective, affordable and safe for the treatment of the abovementioned diseases in order to increase the quality of life and therapeutic possibilities available in view of the increased numbers of cases being registered everyday.

Considering the pharmacological potentials of PCO - C extracted from carnauba wax powder and the absence of studies investigating its toxicity, the present study aimed to evaluate PCO - C serum toxicological parameters, biochemical and hematological profiles, as well as mutagenicity and genotoxicity activities using cells extracted from mice.

2. Materials and Methods

The present manuscript is characterized as experimental and quantitative.

Carnauba wax powder was kindly provided by *Pontes Indústria de Cera Ltda*. First, 100 g of carnauba wax powder were mixed with 300 mL of ethyl acetate and 700 mL of hexane. The mixture was subsequently stirred for 30 min and then filtered in qualitative filter paper (40 × 40 cm and pore size 26 µm, Prolab®). The filtrate material was concentrated in a rotary vacuum evaporator, producing a yellowish compound named PCO - C, which was later analyzed by infrared (IR) spectroscopy and proton nuclear magnetic resonance (^{1}H-NMR).

The IR spectra of the compound were assessed while using a VERTEX 70v (Bruker) Fourier transform infrared (FT-IR) spectrophotometer in vacuum. The samples were deposited on the diamond crystal and the spectra were obtained within the absorbance range of 600 to 4000 cm^{-1} with a 4 cm^{-1} resolution in attenuated total reflectance (ATR) mode.

The ^{1}H-NMR spectra were recorded at 500.13 MHz with a Bruker Avance DRX-500 Spectrometers® while using a 5-mm dual probe with the tetramethyl silane (TMS) signal as the internal standard and $CDCl_3$ as the solvent.

The dose (PCO - C – 500 mg/Kg) employed in the present study was selected in consonance with previous studies (Rodrigues et al., 2014; Freitas et al., 2016; Arruda-Filho et al., 2017; Guedes et al., 2011). In addition, this dose was selected because the studies mentioned have shown satisfactory results against chronic diseases.

2.1 Animals

Male (30-45 g) and female (25-35 g) Swiss mice were used in the present study (n=40), which were randomly assigned. After a period of 5 days of acclimation, the experimental protocol was started and performed during 90 consecutive days through orally (gavage) administration of PCO - C at the dose of 0.5 g / kg. The negative control group was treated only with the PCO - C dilution vehicle (Tween 80 - 4% in distilled water). All experimental procedures with the animals were approved by the Research Ethics Committee of the Federal University of Ceara under number 90/10.

2.2 Determination of body weight gain, the relative weight of organs and histopathological analyses

The animals were weighed at the beginning and at the end of the experiment and kept under clinical observation daily. After the last day of treatment, the animals were euthanized by cervical dislocation and their organs (lung, spleen, kidneys, liver, stomach and heart) were dissected for evaluation of relative weight and part of them were submitted to histopathological analysis.

2.3 Determination of the biochemical profile

The animals were fasted for 8 hr before euthanasia. The mice were anesthetized with halothane and the blood was collected (via plexus-orbital) with heparinized pipettes and sterile tubes. Plasma was obtained by centrifugation (5000 g / 10 min) and subjected to standardized commercial kits (Labtest® and Laborlab®) techniques based on kinetic, enzymatic and colorimetric methods by spectrophotometry. The collected serum was used for the analysis of hepatic function (aspartate aminotransferase, AST, alanine aminotransferase, ALT, blood urea nitrogen), renal (Creatinine and Urea) and total cholesterol.

2.4 Determination of mutagenicity

The femurs of the euthanized animals were removed and the bone marrow collected with the aid of syringe and needle. The collected material was resuspended in fetal bovine serum and transferred to slides (2 per animal). After fixation and staining by the Leishman method, the slides were analyzed using a binocular optical microscope. Micronuclei (MNs) were the typically rounded structures with a diameter of 1/5 to 1/20 of the diameter of the young erythrocytes identified by bluish coloration. A total of 2000 polychromatic erythrocytes were quantified per slide (Heddle et al., 1973). This test had three distinct groups: Negative control, whose treatment was the saline solution; Positive control, whose treatment was cyclophosphamide (25 mg/kg) and; Test sample, PCO - C (500 mg/kg/day).

2.5 Determination of genotoxicity

A total of 200 μL of peripheral blood from each animal was removed and Ficoll was added to allow isolation of LSPH. After being isolated, 20 μL of mononuclear cells were transferred to microtubes containing 110 μL of 0.5% low melting agarose for preparation of the slides. The cells were counted under fluorescence microscopy after staining with ethidium bromide. The analysis was performed according to the pattern of scores previously determined by the size and intensity of the tail of the comet (Shi et al., 2014). 50 comets / microscopic slide were counted and classified according to the percentage of DNA in the tail of the comet, indicating the degree of DNA damage, where 0 = no damage (<5%), 1 = low damage level (5- 20%), 2 = medium damage level (20-40%), 3 = high damage level (40-95%) and 4 = total damage (95%).

The following formula was used to calculate the DNA damage index (DI): DI = 400 - Σ Scores. Data were analyzed by means ± standard error of the mean. Significant differences between groups were calculated by analysis of variance (ANOVA) followed by Student Newman-Keuls ($p < 0.05$) using the Prism software version 5.0 (GraphPad, Intuitive Software for Science, San Diego, CA).

2.6 Statistical analysis

The experiments were analyzed by means ± standard error of the mean. Significant differences between groups were calculated by analysis of variance (ANOVA) followed by Student Newman-Keuls ($p < 0.05$) using the Prism software version 5.0 (GraphPad, Intuitive Software for Science, San Diego, CA).

3. Results

3.1 Identification of p-Methoxycinnamic acid diesters extracted from carnauba wax powder

The results from the infrared (IR) spectroscopic analyses and ^{1}H-NMR enabled the identification of PCO - C as 4-methoxycinnamic acid diester (Table 1), according to the study that was reported by Vandenburg and Wilder (1967).

Table 1. Chemical shift, ^{1}H-NMR spectral data of 4-methoxycinnamic acid diester (PCO - C) with the corresponding numbering.

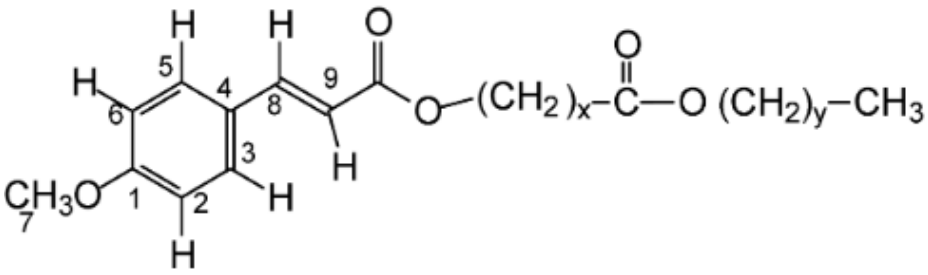
Chemical shift (ppm)	Assignments
7.62	8 (d, 1 H, 16 Hz)
7.45	5, 3 (m, 2 H)
6.85	6, 2 (m, 2 H)
6.33	9 (d, 1 H, 16 Hz)
3.83, 3.90	7 (s, 3 H)

x + y = 58 mean value.
Source: Author himself.

The aforementioned table shows the signs and the chemical shift (ppm) of each hydrogen present in the chemical compound used in the present study, resulting in the possibility of identifying the material used in the pharmacological tests.

The analysis of PCO - C and infrared absorption spectroscopy identified the presence of absorption bands characteristic of ester (1738, 1717, and 1169 cm−1), unsaturated (163 and, 930 cm−1), p-substituted aromatic (830 cm−1), and p-methoxy aromatic (1020 cm−1) functional groups. The ^{1}H-NMR spectrum and its expansion confirmed that PCO - C is a 4-methoxycinnamic acid ester, according to the spectroscopic data and the structure (Figure 1 and Table 1).

Figure 1. Structural representation of 4-methoxycinnamic acid diester (PCO - C).



Source: Author himself.

3.2 Body mass of animals

As shown in Table 2, the PCO - C in the tested dose was not able to promote significant changes ($p > 0.05$) in the body mass of the male and female mice, neither interfered in the relative mass gain, when compared with the animals of the group NC.

Table 2. Effect of PCO - C on mass and relative gain of male and female mice.

	NC		**PCO – C (500 mg / Kg)**	
Male animals				
Body mass (g)	Initial weight	Final weight	Initial weight	Final weight
	31.0 ± 1.0	43.0 ± 1.22	30.5 ± 0.9	42.0 ± 0.8
Relative mass gain (g)	12.0 ± 2.0		11.5 ± 1.3	
Female animals				
	Initial weight	Final weight	Initial weight	Final weight
Body mass (g)	33.0 ± 1.3	35.0 ± 1.67	29.5 ± 0.9	30.0 ± 0.7
Relative mass gain (g)	2.0 ± 2.26		0.5 ± 0.9	

NC, Normal control; PCO – C (500 mg / Kg). Values are given as the mean ± SEM of 10 mice per group. To analyze the significance of the differences between the groups was used analysis of variance (ANOVA) followed by the Newman-Keuls comparison test. Relative gain of the mass of animals was performed by comparison between the beginning and the end of the experiment.
Source: Author himself.

3.3 Relative organs weight of treated male and female animals

In the present study, as shown in Table 3, PCO - C (500 mg/kg) showed no statistically significant changes ($p > 0.05$) in the relative mass of the main susceptible organs of the subchronic toxicity test, when compared to animals in the NC group.

Table 3. Effect of PCO - C on main organs susceptible to changes in the subchronic toxicity test in male and female mice.

	Male Swiss mice		**Female Swiss mice**	
	NC	PCO – C (500 mg / Kg)	NC	PCO – C (500 mg / Kg)
Lung	0.57 ± 0.04	0.56 ± 0.03	0.69 ± 0.07	0.77 ± 0.09
Spleen	0.2 ± 0.02	0.2 ± 0.01	0.4 ± 0.06	0.51 ± 0.15
Kidney	1.48 ± 0.1	1.53 ± 0.09	1.37 ± 0.1	1.71 ± 0.1
Liver	4.16 ± 0.1	3.91 ± 0.09	4.42 ± 0.2	4.52 ± 0.2
Stomach	0.9 ± 0.07	0.98 ± 0.03	1.16 ± 0.1	1.31 ± 0.1
Heart	0.5 ± 0.04	0.5 ± 0.02	0.4 ± 0.04	0.5 ± 0.03

NC, Normal control; PCO – C (500 mg / Kg). Values are given as the mean ± SEM of 10 mice per group. To analyze the significance of the differences between the groups was used analysis of variance (ANOVA) followed by the Newman-Keuls comparison test.

Source: Author himself.

3.4 Biochemical profile

Initially, the effects of PCO - C (500 mg/kg) on the main biochemical parameters were investigated and serum levels of total cholesterol, urea, creatinine, alanine and aspartate aminotransferases were measured.

After 90 days of treatment with PCO - C, it was possible to observe that relevant parameters such as total cholesterol, creatinine, ALT and AST did not show significant alterations ($p > 0.05$) when compared to male and female animals of the NC group.

However, significant reductions ($p < 0.05$) were observed in serum urea in male animals (48.0 mg/dl) and females (39.8 mg / dl) receiving 500 mg/kg PCO - C, a mean reduction of 21.7%, in relation to the respective animals of the NC groups (63.8 and 49 mg / dl) (Table 4).

Table 4. Effect of PCO - C on serum markers of male and female mice.

	Male Swiss mice		**Female Swiss mice**	
	NC	PCO – C	NC	PCO – C
TC (mg/dl)	147.2 ± 11.2	149. 6 ± 8.7	119.3 ± 3.7	110.3 ± 3.68
Urea (mg/dl)	63.8 ± 6.4	48.0 ± 1.6*	49.0 ± 2.9	39.8 ± 1.46*
Creat (mg/dl)	0.51 ± 0.04	0.42 ± 0.03	0.34 ± 0.02	0.32 ± 0.03
ALT (U/L)	28.2 ± 2.8	39.4 ± 4.0	32.0 ± 2.1	30.7 ± 2.1
AST (U/L)	61.6 ± 4.3	60.5 ± 4.4	59.7 ± 3.1	55.8 ± 1.92

NC, Normal control; PCO – C (500 mg / Kg); TC, Total cholesterol; Creat, Creatinine; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase. Values are given as the mean ± SEM of 10 mice per group. To analyze the significance of the differences between the groups was used analysis of variance (ANOVA) followed by the Newman-Keuls comparison test. *p<0,05 versus the NC group. Source: Author himself.

3.5 Hematological profile of treated male and female animals

The subchronic toxicity assessment of PCO - C at a dose of 500 mg/kg showed no behavioral changes or mortality in both sexes. In addition, after 90 days of oral supplementation, the hematological parameters evaluated did not differ statistically (p> 0.05) when compared to the animals in the NC group (Table 5), being considered as a strong indicative of hematological normality and low / absence toxicological influence from the PCO - C at the dose tested in the present study.

Table 5. Effect of PCO - C on hematological parameters of male and female mice

	Male Swiss mice		Female Swiss mice	
	NC	PCO – C	NC	PCO – C
Erythrocytes (x 10^6/µL)	8.34 ± 0.2	8.22 ± 0.1	8.3 ± 0.1	8.2 ± 0.1
Hemoglobin (g/dl)	11.3 ± 0.3	11.4 ± 0.2	13.7 ± 0.3	13.9 ± 0.2
Hematocrit (%)	43.0 ± 0.8	44.8 ± 0.9	44.8 ± 0.8	45.2 ± 0.9
MCV[a] (%)	51.6 ± 1.0	52.5 ± 0.5	54.0 ± 0.5	54.5 ± 0.5
MCH[b] (%)	13.6 ± 0.2	13.2 ± 0.1	16.5 ± 0.2	16.7 ± 0.1
MCHC[c] (%)	26.3 ± 0.4	26.1 ± 0.1	30.6 ± 0.2	30.7 ± 0.1
Platelets (10^3/µL)	1293 ± 150.2	1351 ± 72.1	1214 ± 76.9	1543 ± 72.1
Leukocytes totais (10^3/µL)	4.2 ± 0.5	3.1 ± 0.2	3.8 ± 0.8	4.3 ± 0.2
Segmented (%)	22.6 ± 2.4	26.2 ± 1.4	9.8 ± 1.5	13.6 ± 1.4
Lymphocytes (%)	77.4 ± 2.4	72.0 ± 1.4	89.0 ± 1.7	85.4 ± 1.4
Monocytes (%)	0.00 ± 0.00	0.00 ± 0.00	0.8 ± 0.2	0.6 ± 0.2
Eosinophils	0.00 ± 0.00	0.01 ± 0.01	0.4 ± 0.2	0.4 ± 0.1

NC, Normal control; PCO – C (500 mg/Kg); MCV, Mean Corpuscular Volume; MCH, Mean Corpuscular Hemoglobin; CHCM, Mean Corpuscular Hemoglobin Concentration Values are given as the mean ± SEM of 10 mice per group. To analyze the significance of the differences between the groups was used analysis of variance (ANOVA) followed by the Newman-Keuls comparison test.
Source: Author himself.

3.6 Histopathological analyzes

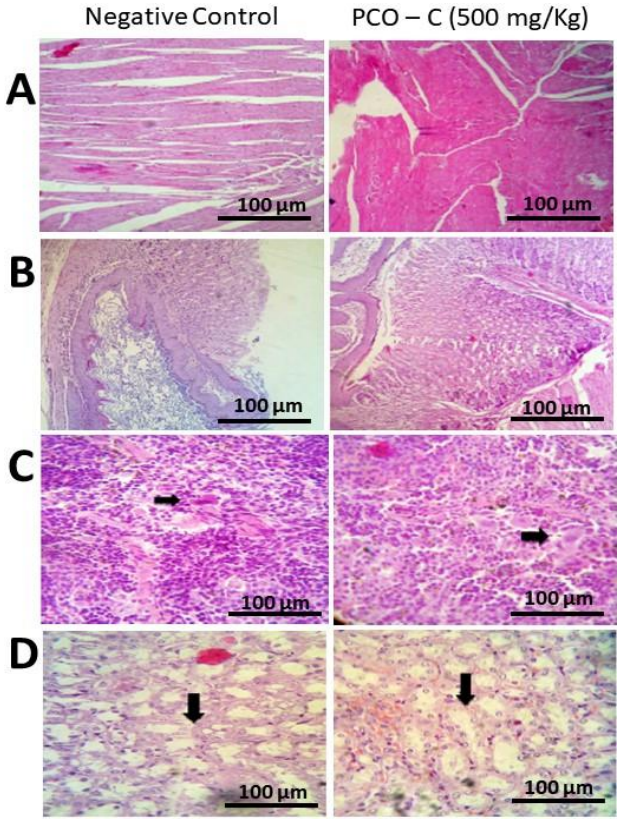
After 90 days of treatment with PCO - C, animals submitted to the subchronic toxicity protocol were euthanized and the organs heart, intestine, spleen, kidneys and were analyzed microscopically.

In the cardiac analysis of the animals of the NC and PCO - C group (Fig. 2A), it was possible to observe the intact cardiac fibers, showing no distortion or signs of cytotoxicity.

Observing the stomach, the present study did not show architectural alterations at the gastroesophageal junction, cardia, body/antrum and pylorus, and did not present epithelial cytotoxicity or inflammation in the groups of animals that received PCO - C or in the control group (Fig. 2B).

Futhermore no architectural changes were observed in the spleen (Figure 2C). In addition, the animals of the NC and PCO - C group showed no evidence of cytotoxicity. According to Figure 2C, there is the presence of extramedullary hematopoiesis in the red pulp of all groups analyzed. However, this change is considered typical in rodents due to compensatory extramedullary hematopoiesis during their life cycle.

Figure 2. Histological appearance of organs of PCO - C-treated (500 mg/Kg) and control group (Tween 4%).



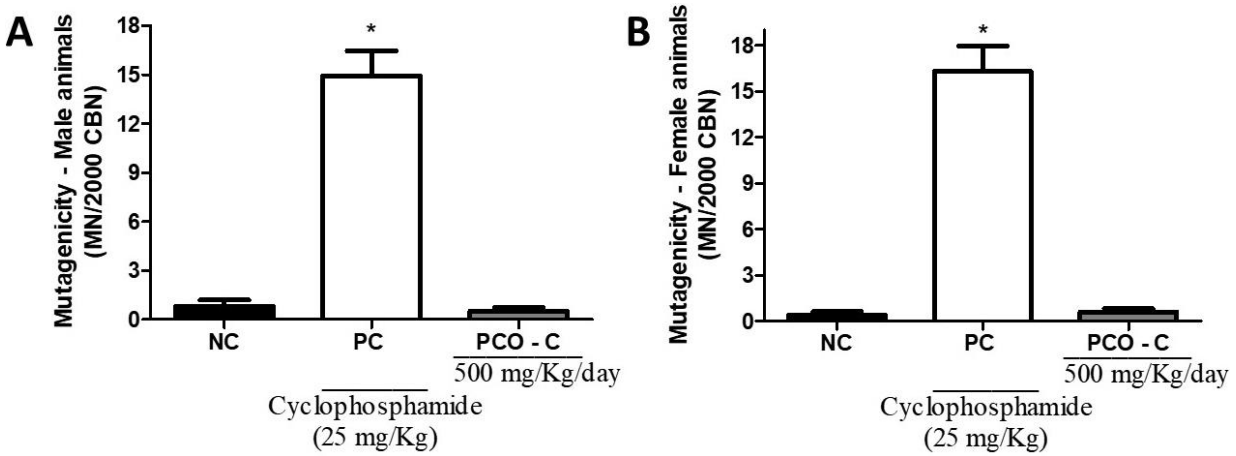
(A) heart; (B) stomach; (C) spleen; (D) kidney. Stain, hematoxylin and eosin; magnification ×200 for A, C and D and ×100 for B; PCO – C, *p*-methoxycinnamic diesters.
Source: Author himself.

In the present study, the microscopic analysis of the kidneys (Fig. 2D) of the animals treated with PCO - C showed preserved tubular and glomerular structure, indicating architectural conservation in this organ. However, slight lymphoplasmacytic inflammation was observed in the animals treated with PCO - C and vehicle.

3.7 Potential of mutagenicity in male and female swiss mice

The results obtained in the analysis of mutagenicity (Figure 3A and 3B) by the micronucleus test of femurs collected from both sexes showed that PCO - C did not present mutagenic effect at the dose tested (500 mg/kg/day) due to similarity ($p > 0.05$) and statistically differed ($p < 0.05$) from the positive group of the test in which cyclophosphamide (25 mg/kg) was used.

Figure 3. Mutagenic effect of PCO - C on male and female swiss animals.

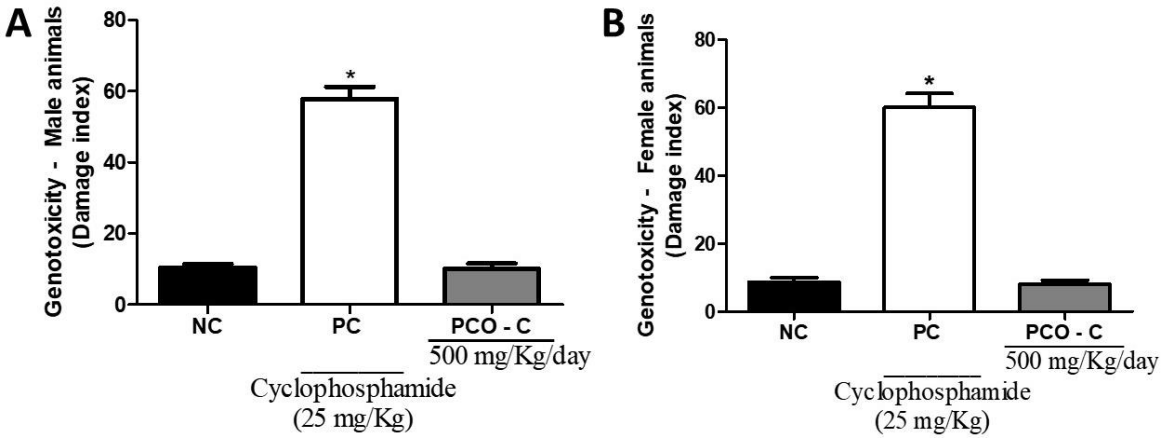


NC, Normal control; PC, Positive control; PCO – C (0.5 g / Kg); MN, micronuclei; CBN, binucleate cells. Values are given as the mean ± SEM of 10 mice per group. To analyze the significance of the differences between the groups was used analysis of variance (ANOVA) followed by the Newman-Keuls comparison test. *p<0,05 versus the NC group.
Source: Author himself.

3.8 Genotoxic effect by the comet test in vivo

In the present study, PCO - C did not present genotoxic activity against HPBLs at the dose tested (500 mg/kg/day), as can be observed in Figure 4.

Figure 4. Genotoxic effect of PCO - C by comet test in male and female animals.



NC, Normal control; PC, Positive control; PCO – C (500 mg / Kg). Values are given as the mean ± SEM of 10 mice per group. To analyze the significance of the differences between the groups was used analysis of variance (ANOVA) followed by the Newman-Keuls comparison test. *p<0.05 versus the NC group.
Source: Author himself.

Therefore, PCO - C was not able to cause significant DNA damage ($p < 0.05$) in male animals (Figure 4A) (10.1 ± 1.4) and female animals (Figure 4B) (8.0 ± 1.2) when compared to the male and female group of the NC group (10.4 ± 1.5 and 8.7 ± 1.2, respectively) who received only vehicle. In addition, animals of the PCO - C group differed statistically ($p <0.05$) from the animals receiving cyclophosphamide (25 mg/kg) (57.8 ± 3.4 and 60.0 ± 4.1, respectively), the latter being a highly genotoxic substance.

4. Discussion

Despite several pharmacological studies with materials extracted from plants or parts of them, few data are available in the scientific literature to determine the safety of these compounds. In the search for the safety of plant products, toxicological studies should be performed using various experimental models to detect toxicity and establish criteria for the selection of a dose that can be considered safe and effective in humans. At various times, toxicity in animals or people occurs through biochemical, hematological, gastrointestinal, or cardiovascular adverse effects and some deleterious effects are correlated with structural rearrangements of the genome caused by different types of DNA damage (Shi et al., 2014).

According to Wolfmeier et al. (2005), carnauba wax is composed of a heterogeneous mixture of esters, free alcohols, aliphatic acids, aromatic acids, ω-hydroxycarboxylic free

acids, hydrocarbons and diols triterpenes. Among these, esters are the major components, corresponding to over 80% of the composition, with a predominance of aliphatic esters and diesters of cinnamic acid (Ullmann's, 1997, Vandenburg and Wilder, 1970).

These components contribute to the low polarity of carnauba wax and one of the highest melting point and strength among the waxes (European Food Safety Authority, 2012), favoring its commercial applicability (Freitas et al., 2019).

The use of carnauba wax in foods is permitted by the Food and Drug Administration (FDA) as well as by the European Food Safety Authority (EFSA) and it can be inserted into foods or emulsions for coating fruits or vegetables that contribute to prevent premature ripening, loss of water, exposure to microorganisms, among other purposes (FDA, 1983; EFSA, 2012). These associations mentioned above recognize carnauba wax as a generally recognized component as safe (GRAS), provided it is in the recommended proportions. Recently, an extensive review was published by Freitas et al., (2019) in which the authors cite the various insertions of carnauba wax in the food sector.

PCO - C is a cinnamic diester commonly found in carnauba wax (Wolfmeier et al., 2005), in which it has been associated with the promising pharmacological properties previously reported by our research group due to its hypocholesterolemic (Guedes et al., 2011; Arruda-Filho et al., 2017), hypoglycemic (Rodrigues et al., 2014) and antioxidant (Freitas et al., 2016). We can highlight that lower doses of PCO - C were used in these previous studies and, in relation to the present study, the treatment of 500 mg/kg used corresponds to the intake of 35 g of PCO - C daily. However, the subchronic toxicological tests performed in this study are relevant and complementary to data previously published.

Subchronic toxicity studies evaluate the undesirable effects of continuous or repeated exposure of compounds extracted from plants in the average life of experimental animals, such as rodents. They provide information on target organ toxicity and are designed to identify and quantify levels of adverse effects (Ping et al., 2013).

Interestingly, the PCO - C employed in the present study was not able to significantly alter the body mass of animals of both sexes. In addition, PCO - C did not cause detectable changes in the relative / absolute mass of the organs, as shown in Tables 2 and 3. Another relevant data refers to the absence of behavioral changes, mortality, atrophy, hypertrophy or swelling. The relative weight of the organs has been observed in several toxicity tests because it is considered an indicator sensitive to harmful changes caused by exogenous substances (Zhang et al., 2016). The result of our study revealed that essential organs such as heart, liver, spleen, stomach, kidneys and lungs were not adversely affected and showed no clinical signs

of toxicity. Together, these data indicate that PCO - C has not been able to promote serious pathological changes and it can be considered a strong indication of low or no toxicity of said sample at the dose tested (500 mg/kg) (Kwo, Cohen & Lim, 2017).

The biochemical profile of the animals presented in Table 4 shows that daily administration of PCO - C in the dose tested did not promote significant alterations ($p > 0.05$) in the serum concentration of hepatic enzymes (ALT and AST), creatinine and total cholesterol in male and female animals. ALT and AST are considered important necrosis/lesion markers in hepatic cells, as well as the presence of alterations in the permeability of hepatocytes by referred elevations. In addition, the increased concentration of one of the above serum enzymes is related to the number of damaged cells. The hepatocyte lesion is usually associated with cholestasis, regardless of the possible causes (inflammatory, degenerative or neoplastic), since the bile canaliculi may become obstructed as a consequence of liver cell dilation (Messias et al., 2010). However, oral administration of PCO - C showed the dose of 500 mg/kg of PCO - C was not toxic to the liver during all experiment.

Exceptionally, serum urea from animals of both sexes, an important byproduct of protein degradation (Paim et al., 2017), showed a significant reduction ($p < 0.05$) when compared to the initial values. These markers are important toxicological parameters since the excess can be indicative of tissue injury, renal and hepatic metabolic alterations or dysfunctions caused by oxidative stress. In addition, elevated levels of urea and creatinine are considered indicative severe of renal damage (Naqshbandi et al., 2012), in contrast to the results shown in our study. One of the possible causes for the aforementioned reduction may be due to the influence of the cinnamic acids, abundantly mentioned in the scientific literature, in the increase of mRNA synthesis of the RB1CC1 and MAP1LC3B genes in renal tissue due to its capacity to induce autophagy in the kidneys and to reduce indices of biomarkers directly related to oxidative stress, including urea (Mamal et al., 2012; Matboli et al., 2017).

The results obtained in Table 5 demonstrated that oral administration of p-methoxycinnamic diesters during the subchronic toxicity test did not produce adverse changes in red blood cells of the male and female mice. Hematological evaluation represents an essential tool in the investigation of possible deleterious effects of chemical compounds extracted from plants on the health status of animals in toxicological tests due to the evaluation of the diagnosis of several reactions. This approach can also be used to explain the relevant blood functions of a plant extract or its products (Zhang et al., 2016).

The leukogram results (Table 5) also show that there was no significant difference ($p > 0.05$) observed between the control group and the experimental group of both sexes. These data are relevant because they indicate that the immune system did not mobilize an increase in the activity of the cellular defense mechanisms and consequently did not identify the test sample extracted from the carnauba wax powder as antigen, as shown by the maintenance of the levels of monocytes, leukocytes, lymphocytes and other components that are directly related to inflammatory processes or immunomodulatory action from the activation of macrophages (Pinto et al., 2010).

The results of the histopathological study provide evidence corroborating with the data found in the biochemical, hematological and body mass profiles found in our research. No severe histopathological abnormalities were detected in the heart, stomach, spleen and kidneys of animals that were orally supplemented with PCO - C at a dose of 500 mg/kg for 90 days.

The histopathological sections shown in Figure 2 indicate the absence of severe lesions and showed a standard structural architecture for all organs analyzed related to the 90 days of PCO - C treatment. Although an extramedullary hematopoiesis in the spleen and slight renal lymphoplasmacytic inflammation was identified in the animals treated with PCO - C, it was also observed that the animals of the control group had the same changes mentioned in our study, indicating absence of an abnormality caused by PCO - C at a dose of 500 mg/kg.

Recent studies with cinnamic acid derivatives demonstrate a high potential for this category of chemical compounds. Several beneficial effects have been attributed compound similar to PCO – C due to the presence of electron-donating substituents present on the aromatic ring of referred compound (hydroxyl and / or methoxyl), in addition to the carboxyl radical with a double bond unsaturation, in which it provides more attack mechanisms to free radical free sites and act as an anchor in the lipid bilayer. Another relevant important data related to PCO - C is its esterified form, in which potentiates its beneficial pharmacological effects (Jakovetić et al., 2013). In this way, we can attribute the protective effects, maintenance of biochemical and hematological parameters, consistent with the data found in the histopathological analyzes found in the present study, to the chemical characteristics previously reported in the scientific literature (Guedes et al., 2011; Rodrigues et al., 2014; Freitas et al., 2016; Arruda-Filho et al., 2017).

The results obtained in the tests of genotoxicity and mutagenicity showed that the animals that received a high dose of PCO - C (500 mg/kg) had no significant difference when compared to NC group and statistically differed ($p < 0.05$) of animals from PC group.

Furthermore, it is essential to note that PCO - C was not able to induce an increase in mutagenicity/genotoxicity, observed in animals of the PC group that received cyclophosphamide, an important inducer of DNA damage (Kour et al., 2017), and showed that PCO - C was not mutagenic in said analysis because of the absence of structural integrity of DNA damage or changes in micronuclei.

According to the results from the present study, p-methoxycinnamic acid diesters extracted from carnauba wax powder (PCO - C) showed no cytotoxic effect against healthy human cells, which has also been reported by several authors who studied cinnamic acid and its derivatives (Sova et al., 2013; Shi et al., 2014). Importantly, cinnamic acid and several of its esters showed no cytotoxicity on healthy human cells but showed a selective cytotoxic effect on malignant cell lines as reported by Sova et al.,(2013).

The absence of mutagenicity and genotoxicity found in our study can be justified by the antioxidant effect of the hydroxyl radicals or the esters present in the cinnamic acid derivatives (Taner et al., 2016). These results are relevant because inhibition of DNA damage and / or repair activity caused by antioxidant compounds is an important tool for the prevention of mutations and carcinogens (Kour et al., 2017).

Based on our results, oral administration of PCO - C appears to be well tolerated in mice. PCO - C seemed to have no clinically significant toxic effects on the physiological and biochemical functions of animals of both sexes after the subchronic toxicity test. In summary, PCO - C presented consistent effects on the maintenance of several parameters evaluated, such as biochemical, hematological, relative organ masses, histological, mutagenic or genotoxic, and did not present behavioral changes or deaths in treated animals during 90 days of the experiment. Although the effects studied in the animals are satisfactory and suggest no toxicity at the dose tested, it is necessary to extrapolate the toxicity test in humans in which clinical studies are necessary to define the safe dose in humans.

5. Conclusion and Suggestions

In conclusion, infrared spectrometry and ^{1}H-NMR further characterized the *p*-methoxycinnamic acid diester from carnauba wax powder (PCO - C) and subjected to toxicological tests.

The PCO - C did not present deleterious effects on the blood parameters (hematological and biochemical), histopathological and relative mass of the organs. In addition, PCO - C at the dose of 500 mg/kg was not able to promote mutagenic and genotoxic changes in the in

vivo test. Although the compound does not present evidence of oral subchronic toxicity in mice, studies in humans are required to ratify and select the best dose for administration and finally to obtain the exceptional pharmacological effects previously reported.

In view of the results obtained in the present manuscript, it can be suggested that authorization can be requested from the competent consortia for the start of tests on human beings, since the present manuscript and the others already published with the PCO-C indicate good pharmacological activity and reliable toxicological security.

References

Albuquerque, U. P., Medeiros, P. M., de Almeida, A. L. S., Monteiro, J. M., de Freitas Lins Neto, E. M., de Melo, J. G., & dos Santos, J. P. (2007). Medicinal plants of the caatinga (semi-arid) vegetation of NE Brazil: A quantitative approach. *Journal of Ethnopharmacology*, 114 (1), 325–354.

Ayres, M. C. C., Brandão, M. S., Vieira-Júnior, G. M., Menor, J. C. A. S., Silva, H. B., Soares, M. J. S., & Chaves, M. H. (2018). Atividade antibacteriana de plantas úteis e constituintes químicos da raiz de *Copernicia prunifera*. *Revista Brasileira de Farmacognosia*, 18 (3), 35-39.

European Food Safety Authority. (2012). Scientific Opinion on the re-evaluation of carnauba wax (E 903) as a food additive EFSA Journal, 10 (2012), 10.2903/j.efsa.2012.2880

FDA. Food and Drugs Chapter 9 - federal food, drug, and cosmetic act, Subchapter II - Definitions, Sec. 321 – Definitions; generally, Code of Federal Regulations, Titel 21, Sec. 321 § (1983). https://doi.org/CFR.

Filho, A. C. V., Rodrigues, P. A. S., Benjamin, S. R., Paim, R. T. T., Holanda, M. O., Silva, J. Y. G., Guedes, M. I. F. (2017). Hypolipidemic activity of P-methoxycinnamic diester (PCO - C) isolated from *Copernicia prunífera* against Triton WR-1339 and hyperlipidemic diet in mice. *Environmental Toxicology and Pharmacology*, 56 (2017), 198–203.

Freitas, C. A. S., de Sousa, P. H. M., Soares, D. J., da Silva, J. Y. G., Benjamin, S. Rathinaraj, & Guedes, M. I. F. (2019). Carnauba wax uses in food - a review. *Food Chemistry*, 291 (2019), 1-38

Freitas, C.A.S., Vieira, Í.G.P., Sousa, P.H.M., Muniz, C.R., Gonzaga, M.L.D.C. &, Guedes, M.I.F. (2016). Carnauba wax p-methoxycinnamic diesters: Characterisation, antioxidant activity and simulated gastrointestinal digestion followed by in vitro bioaccessibility. *Food Chemistry*, 196 (2016), 1293–1300.

Guedes, M. I. F., Alves, C. R., Vieira, I. G. P., Almeida, L. M., Mendes, F. N. P., Duarte, L. S. F., & Arruda Filho, A. C. V. Processo de produção, uso e composição farmacêutica compreendendo compostos obtidos a partir de cera de carnaúba (2011). [Production process, use and pharmaceutical composition of carnauba wax-derived products]. Patente BR 2011/1012429-2

Jakovetić, S. M., Jugović, B. Z., Gvozdenović, M. M., Bezbradica, D. I., Antov, M. G., Mijin, D. Ž., & Knežević-Jugović, Z. D. (2013). Synthesis of Aliphatic Esters of Cinnamic Acid as Potential Lipophilic Antioxidants Catalyzed by Lipase B from *Candida antarctica*. Applied Biochemistry and Biotechnology, 170(6), 1560–1573.

Kour, J., Ali, M. N., Ganaie, H. A., & Tabassum, N. (2017). Amelioration of the cyclophosphamide induced genotoxic damage in mice by the ethanolic extract of E*quisetum arvense*. *Toxicology Reports*, 4(2), 226–233.

Lorenzi, H., Noblick, L., Kahn, F., Ferreira, E. (2010). Brazilian flora Lorenzi: *Arecaceae* (palms). Instituto Plantarum de Estudos da Flora. *Nova Odessa*, 368 (2), 43-9.

Mamal, E., Basar, M., Uzun, H. & Seckin, I. Caffeic Acid Phenethyl Ester Prevents Mesengial Cell Apoptosis by Suppressing zp38MAPK Signal. J.Cytol. Histol. 3(2012), 4451-4475.

Matboli, M., Eissa, S., Ibrahim, D., Hegazy, M. G. A., Imam, S. S., & Habib, E. K. (2017). Caffeic Acid Attenuates Diabetic Kidney Disease via Modulation of Autophagy in a High-Fat Diet/Streptozotocin- Induced Diabetic Rat. Scientific Reports, 7(2017), 11-24.

Messias, J. B., Caraciolo, M. C. M., Oliveira, I. M. de, Montarroyos, U. R., Bastos, I. V. G. A., Guerra, M. de O., & Souza, I. A. (2010). Avaliação dos parâmetros hematológicos e bioquímicos de ratas no segundo terço da gestação submetidas à ação do extrato metanólico de *Cereus jamacaru* DC., *Cactaceae*. *Revista Brasileira de Farmacognosia*, 20(2010), 478–483.

Naqshbandi, A., Khan, M.W., Rizwan, S., Rehman, S.U., Khan, F. (2012). Studies on the protective effect of dietary fish oil on cisplatin-induced nephrotoxicity in rats. *Food Chem Toxicol*, 50(22), 265-273.

Organization for Economic Cooperation and Development. Guideline 425 for testing of chemicals. *Paris: OECD*, 1996.to

Ola, M. S., Ahmed, M. M., Ahmad, R., Abuohashish, H. M., Al-Rejaie, S. S., & Alhomida, A. S. (2015). Neuroprotective Effects of Rutin in Streptozotocin-Induced Diabetic Rat Retina. *Journal of Molecular Neuroscience*, 56(15), 440–448.

Paim, R.T.T., Benjamin, S.R., Rondina, D., Marques, M.M.M., de A. Viana, D., da C. Gonzaga, M.L., Vieira, Í.G.P., Mendes, F.N.P., Rodrigues, P.A.S., & Guedes, M.I.F. (2017). Antihypercholesterolemic effects of fruit aqueous extract of *Copernicia prunifera* (Miller) H. E. Moore in mice diet-induced hypercholesterolemia. *Evidence-Based complement. Altern. Medicine*, 2017(2017), 1–15.

Ping, K. Y, Darah, I., Chen, Y., Sreeramanan, & S., Sasidharan, S. (2013). Acute and subchronic toxicity study of *Euphorbia hirta* L. methanol extract in rats. *Biomed. Res. Int*., 2013(3), 1-14.

Pinto, M. C. L., Melo, M. M., Costa, M. E. R., & Labarrere, C. R. (2010). Hematological and biochemical profiles of rats submitted to experimental poisoning with *Tityus serrulatus* venom. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, 62(10), pp. 350–356.

Rodrigues, P. A. S., Guedes, M. I. F., Marques, M. M. M., Silva, I. N. G. Da, & Vieira, Í. G. P. (2014). Hypoglycemic activity of *Copernicia cerifera* mart. Leaf powder extract in the

treatment of alloxan-induced diabetic mice. *International Journal of Pharmacy and Pharmaceutical Sciences*, 6(14), 115–118.

Shi, H., Xie, D., Yang, R., & Cheng, Y. (2014). Synthesis of caffeic acid phenethyl ester derivatives, and their cytoprotective and neuritogenic activities in PC12 cells. *J Agric Food Chem*, 62(2), 5046-5053.

Sova, M., Zizak, Z., Stankovic, J., Prijatelj, M., Turk, S., Juranic, Z., Mlinaric-Rascan, I., & Gobec, S. (2013). Cinnamic Acid Derivatives Induce Cell Cycle Arrest in Carcinoma Cell Lines. *Medicinal Chemistry*, 9(2013), 633–641.

Taner, G., Özkan Vardar, D., Aydin, S., Aytaç, Z., Başaran, A., & Başaran, N. (2016). Use of *in vitro* assays to assess the potential cytotoxic, genotoxic and antigenotoxic effects of vanillic and cinnamic acid. *Drug and Chemical Toxicology*, 40(2016), 183–190.

Theodotou, M., Fokianos, K., Mouzouridou, A., Konstantinou, C., Aristotelous, A., Prodromou, D., Chrysikou, A. (2016). The effect of resveratrol on hypertension: A clinical trial. *Experim. and Therap. Medic*., 13 (6), 295–301.

Ullmann's Encyclopedia of Industrial Chemistry, 5th Edition VCH: Weinheim, Germany. 1996/1997. Section A, 28 vols. Section B, 8 vols. DM 19 400 Organic Process Research & Development, 1 (1997), 391-392.

Vandenburg, L. E., & Wilder, E. A. (1970). The structural constituents of carnauba wax. *J. Am. Oil Chem. Soc*., 47(1), 514-518.

WHO. (2017) | Noncommunicable diseases. WHO. Doi:/entity/mediacentre/factsheets/fs355/en/index.html

Percentage of contribution of each author in the manuscript

Natália do Vale Canabrava: 11.1%

José Ytalo Gomes da Silva: 11.1%

Bruno Bezerra da Silva: 11.1%

Marcelo Oliveira Holanda: 11.1%

Carla Laine Silva Lima: 11.1%

Eridan Orlando Pereira Tramontina Florean: 11.1%

Icaro Gusmão Pinto Vieira: 11.1%

Márcia Maria Mendes Marques: 11.1%

Maria Izabel Florindo Guedes: 11.1%